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Expansion of αβ T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus

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SUMMARY

A large proportion of intestinal intraepithelial lymphocytes (IEL) comprises αβ and γδ T-cell receptor (TcR)-bearing T cells. The numbers of αβ and γδ IEL are reported to be very different between germ-free and conventional microbial conditions. In this study, we investigated the kinetics of both types of TcR-bearing cells after microbial colonization in germ-free mice and the influence of thymus deprivation on IEL populations during the microbial association process. Immediately after association with microbes in germ-free animals, the number of αβ TcR-bearing IEL gradually increased. Fourteen days after microbial association the number of αβ IEL equalled that of γδ TcR-bearing IEL. Approximately 1 month after microbial association, the number of αβ IEL was several times greater than that of γδ IEL, having almost reached the level in conventional mice reared in a conventional animal room after birth. On the other hand the number of γδ IEL hardly changed throughout this microbial association process. Two-colour analysis involving anti-αβ TcR and anti-Lyt-2 or Lyt-3 antibodies showed that the major fraction of IEL that increased after microbial association comprised αβ TcR-bearing T cells expressing CD8 antigen composed of a homodimer of α-chains, which was not detected in other gut associated-lymphoid tissues (GALT) such as Peyer's patch, mesenteric lymph node and lamina propria tissue. The number of αβ T cells in these GALT increased within 1 week more quickly than that of IEL. The increase in αβ IEL after microbial association was not prevented by thymectomy. These results strongly suggest that the progenitors of αβ TcR-bearing IEL expand outside the thymus in response to microbial colonization in germ-free mice.

INTRODUCTION

Intestinal intraepithelial lymphocytes (IEL) are considered to play an important role together with intestinal epithelial cells in the first defence line against micro-organisms in the alimentary tract, although the physiological function of IEL remains obscure. Most IEL express αβ T-cell receptor (TcR) or γδ TcR in combination with CD8 molecules. It has been reported that the CD8 molecule in most IEL is composed of a homodimer of α-chains and a heterodimer of α- and β-chains.[1] It has been clarified that γδ and αβ IEL express CD8 molecules consisting of an α homodimer and an α homodimer and an αβ heterodimer, respectively.[2]

Attention is now focused on where IEL differentiate and how they populate the intraepithelial space in the intestine. In particular, γδ IEL are considered to differentiate extrathymically since athymic nude mice have a substantial population of γδ IEL.[3] On the other hand, αβ IEL are scarcely detected in athymic nude mice.[2,4] Recently, it was also shown that αβ IEL were greatly reduced in β_2-microglobulin-deficient mice, although the number of γδ IEL showed no change.[5] However, it was postulated that a proportion of αβ TcR IEL also differentiate extrathymically, because of the absence of negative selection of Vβ6-, and Vβ3- and Vβ11-bearing TcR in AKR/J and BALB/c mice, respectively,[6,7] and the appearance of αβ IEL in old mice (more than 1 year old).[7] Thus, controversy exists regarding the thymus dependency of αβ IEL.

There is a great difference in the number of αβ IEL between germ-free and conventional mice.[8] Therefore, conventionalization of germ-free mice is a good means of exploring the response mechanism of αβ IEL. The cytotoxicity of IEL was reported to be caused by microbial colonization in the host animal in accordance with the expression of Thy-1 antigen.[9]

In the present study, we investigated the kinetics of each phenotype of IEL after microbial colonization in germ-free mice. In addition, we examined the effect of thymus deprivation on the responses of αβ and γδ IEL to microbial association to clarify the thymus dependency of the expansion of αβ IEL.

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MATERIALS AND METHODS

Mice

BALB/c germ-free (GF) mice (13–14 weeks old) and age-matched conventional mice were used in this study. GF mice were kept in our Institute and checked for contamination by micro-organisms. Conventional mice were purchased at the age of 8 weeks from Charles River (Tokyo, Japan) and reared in the conventional animal room in our Institute.

Inoculation of micro-organisms

GF mice were moved from a vinyl-isolator to the conventional animal room and associated with intestinal micro-organisms by oral administration of a faecal suspension (1 faecal pellet/mouse) freshly isolated from conventional mice (conventionalization). We confirmed that the numbers of Enterobacteriaceae, lactobacilli, streptococci, Bacteroidaceae, staphylococci, Bacillus and Funji reached the level in conventional mice within 1 week after conventionalization.

Thymectomy

Immediately after removal of GF mice from a vinyl-isolator, thymectomy was performed under sterile conditions. A sham operation was performed in the same way as for thymectomy except for removal of the thymus. Approximately 2 hr after the operation, the mice were conventionalized as described above.

Preparation of IEL

After cervical dislocation of the mice, their whole small intestines were removed. After removal of visible Peyer's patches (PP), the intestinal contents were thoroughly washed out with phosphate-buffered saline (PBS). Then the small intestine was cut into 0·5–1·0 cm pieces. These pieces were incubated in medium H2387 (Gibco, Grand Island, NY) containing 0·45 mM dithiothreitol for 25 min at 25°. Then the medium was replaced with H2387 medium containing 0·45 mM dithiothreitol and 2 mM ethylendiaminetetraacetate (EDTA). After incubation for 30 min at 37°, the epithelial cells released were collected by centrifugation. Then, after 2 hr incubation in 5% foetal calf serum (FCS)–RPMI-1640, the aggregates were removed and filtered on a cotton column. The resultant cell suspension was applied to a Percoll density gradient (44%) after treatment with lysis buffer for red blood cells . The pellet was recovered as IEL. In the thymectomy experiment, IEL were sedimented on a Percoll density gradient (33%) and then finally recovered from the interface 44%/70% Percoll density centrifugation.

Preparation of PP, mesenteric lymph node (MLN) and lamina propria (LP) lymphocytes

PP and MLN lymphocytes were prepared by passage through stainless steel mesh. LP lymphocytes were obtained by incubation of the residual tissue after IEL isolation in H2387–EDTA medium for an additional 30 min, and RPMI-1640 medium containing 0·6 mg/ml collagenase (Yakult Co., Tokyo, Japan) and 0·1 mg/ml DNase (Sigma Chemical Co, St Louis, MO) for 120 min at 37°. LP lymphocytes, recovered on 45–120 min incubation with collagenase, were used for flow cytometry.

Flow cytometry

The following monoclonal antibodies (mAb) were used. Hamster anti-$\gamma\delta$ TcR mAb (clone 3A10) was kindly provided by Professor S. Tonegawa (MIT, Boston, MA). Fluorescein isothiocyanate (FITC) hamster anti-$\alpha\beta$ TcR (H57-597; Pharmingen, San Diego, CA), FITC hamster anti-CD3-ε (145–2C11; Boehringer-Mannheim-Yamanouchi, Tokyo, Japan), FITC rat anti-Lyt-3 (53-5.8; Pharmingen), phycoerythrin (PE) rat anti-Lyt-2 (53-6.7; Pharmingen), FITC anti-Thy-1.2 (FITC, 010-22F; Meiji Nyugyo Co., Tokyo, Japan), and biotinylated rat anti-CD4 (Rm-4-5; Pharmingen) were purchased from the sources indicated. Polyclonal affinity-isolated FITC anti-mouse IgG + IgA + IgM (Caltag, South San Francisco, CA) was also used to check contamination of B cells. Approximately 2–5×10^5 cells were incubated with an appropriate antibody for 30 min in ice water. If necessary, FITC or PE anti-hamster IgG (Cappel, West Chester, PA) or avidin–FITC (Chemical Credential, Lisle, IL) was used as the second antibody. After staining, cells were washed with Hanks' balanced salt solution (HBSS) and fixed in HBSS containing 1% paraformaldehyde. Cytofluorometric analysis was performed with a fluorescence-activated cell analyser (EPICS ELITE; Coulter Electronics, Hialeah, FL), while dead cells and epithelial cells were excluded by gating forward and side angle scatter. The flow cytometry of PP, MLN or LP lymphocytes was performed by the same gating used in IEL analysis.

Statistics

The difference in the percentage of each phenotype of IEL between GF mice and conventionalized mice was analysed by Student's *t*-test.

RESULTS

Intestinal flora in conventionalized (CV) mice

We confirmed the absence of bacteria in GF mice and that the intestinal flora similar to that in age-matched CV mice was established in the gastrointestinal tract within 1 week after conventionalization of GF mice as descibed elsewhere.[10] We observed that the numbers of Enterobacteriaceae and streptococci in the intestinal content within a few days after conventionalization were greater than in CV mice.

Changes of $\alpha\beta$ and $\gamma\delta$ IEL after conventionalization of GF mice

GF mice were conventionalized at 13 weeks of age by the introduction of a faecal suspension derived from CV mice reared in a conventional animal room. The IEL phenotypes of these conventionalized mice and GF mice immediately before conventionalization were analysed by flow cytometry together with those of age-matched GF and CV mice. Figure 1 shows the per cent composition and the number of each phenotype of IEL, respectively. The number of $\gamma\delta$ IEL remained almost constant throughout the conventionalization process and was not different from that in age-matched CV mice. However, the percentage and the number of $\alpha\beta$ IEL began to increase 4 days after conventionalization. Fourteen days after conventionalization, the number of $\alpha\beta$ IEL was almost equal to that of $\gamma\delta$ IEL. Twenty-eight days after conventionalization, the number of $\alpha\beta$ IEL was approximately three times greater than that of $\gamma\delta$ IEL.

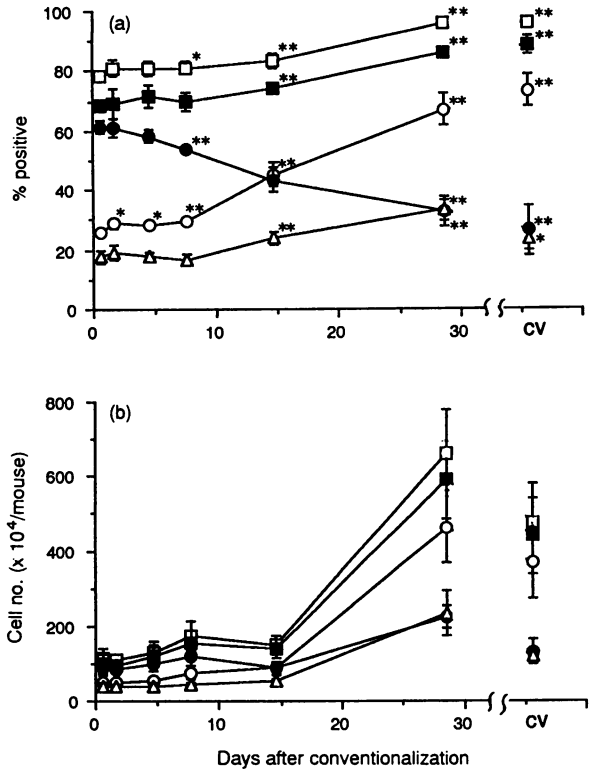

Figure 1. Time-courses of the percentage (a) and number (b) of each phenotype of IEL after microbial colonization in GF mice. The cell number was calculated by multiplying the percentage of each phenotype determined on flow cytometry by the yield of IEL determined on microscopy. GF mice were conventionalized by the introduction of a faecal suspension derived from CV mice. Each point represents the mean ±SD ($n=4$). Values of age-matched CV mice are also shown. CD3 (□); Lyt-2, (■); αβ TcR (○); γδ TcR (●); Thy-1 (△). (a) Significant difference from the value of GF mice: $*P<0.05$; $**P<0.01$.

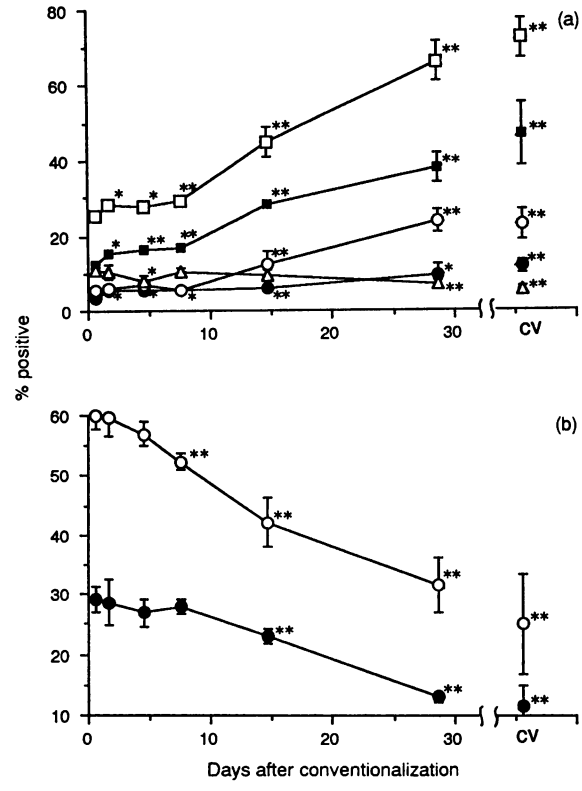

Figure 2. Changes in the percentages of various subsets of αβ TcR- or γδ TcR-bearing IEL during conventionalization of GF mice. The values were obtained from the same experiment as shown in Fig. 1. (a) Total αβ IEL (□); αβ IEL with CD8 consisting of α homodimer (■) or αβ heterodimer (○); double positive IEL (●); CD4 single positive (△). (b) Total γδ IEL (○); double negative IEL (●). Significant difference from the value of GF mice: $*P<0.05$; $**P<0.01$.

The percentage of αβ IEL 28 days after conventionalization was almost the same as that in CV mice, although the number of αβ IEL recovered was slightly greater than that in CV mice. The percentages of CD3-ε$^+$ and CD8$^+$ cells slightly increased from 80 to 90% and 70 to 80%, respectively, after conventionalization. Thy-1$^+$ cells (%) increased twice after conventionalization. CD4$^+$, CD8$^+$ double positive cells (%), a minor fraction of IEL, also increased after it.

Change of αβ IEL with CD8 composed of an α homodimer and an αβ heterodimer after conventionalization of GF mice

αβ IEL express CD8 molecules consisting of an α homodimer and an αβ heterodimer.[2,4] By subtraction of the percentage of αβ TcR$^+$, Lyt-3$^+$ cells from that of αβ TcR$^+$, Lyt-2$^+$ cells, we calculated the percentage of αβ IEL bearing the two types of CD8 molecules. As shown in Fig. 2a, a major fraction of αβ IEL that increased bore CD8 molecules consisting an α homodimer, although both types of CD8-bearing αβ IEL increased after conventionalization. The percentages of double positive and CD4 single positive cells, presumed to bear αβ TcR,[11] slightly increased and decreased, respectively, after that. On the other hand, the percentage of double-negative cells, presumed to be γδ IEL[11] decreased after conventionalization of GF mice (Fig. 2b).

Changes of αβ T cells in GALT after conventionalization of GF mice

The percentages and numbers of αβ TcR in PP, MLN and LP lymphocytes are shown in Fig. 3. Both the percentages and numbers of αβ T cells in GALT increased within 7 days after conventionalization, i.e. earlier than in the case of IEL.

Expression of Lyt-2 and Lyt-3 in IEL and T cells in GALT

The results of two-colour analyses involving anti-Lyt-2 and anti-Lyt-3 of IEL and mononuclear cells in GALT such as PP, MLN and LP 14 days after conventionalization are shown in Fig. 4. IEL contained many Lyt-2 single positive cells, but the lymphocytes in GALT hardly included cells with such a phenotype. Lyt-2 single positive cells consisted of αβ or γδ IEL, as described previously.

Effect of thymectomy on the population of IEL after conventionalization of GF mice

As soon as possible after removal of GF mice from the vinyl-isolator, thymectomy was performed under sterile conditions. The thymectomized and sham-operated mice were conventionalized 2 hr after the operation. Twenty-eight days after conventionalization of GF mice, the total number of IEL recovered and

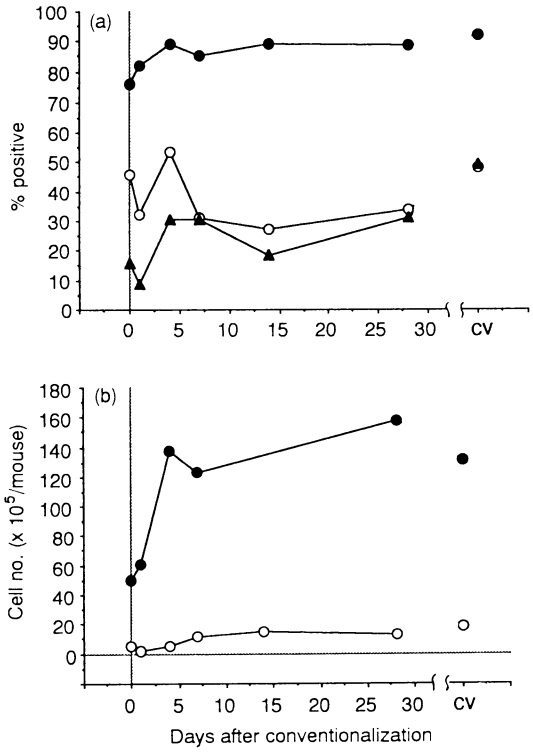

Figure 3. Changes in the percentages (a) and numbers (b) of $\alpha\beta$ TcR T cells in MLN (●), PP (○), and LP (▲) during the conventionalization of GF mice. The data were obtained from the same experiment as shown in Fig. 1. Each point represents the value of four pooled mice. The cell number was calculated by multiplying the percentage of each phenotype determined on flow cytometry by the yield of mononuclear cells determined on microscopy. The cell number of $\alpha\beta$ T cells in LP lymphocytes is not shown here because there was some difference in the efficiency of recovery of lymphocytes between GF and CV mice.

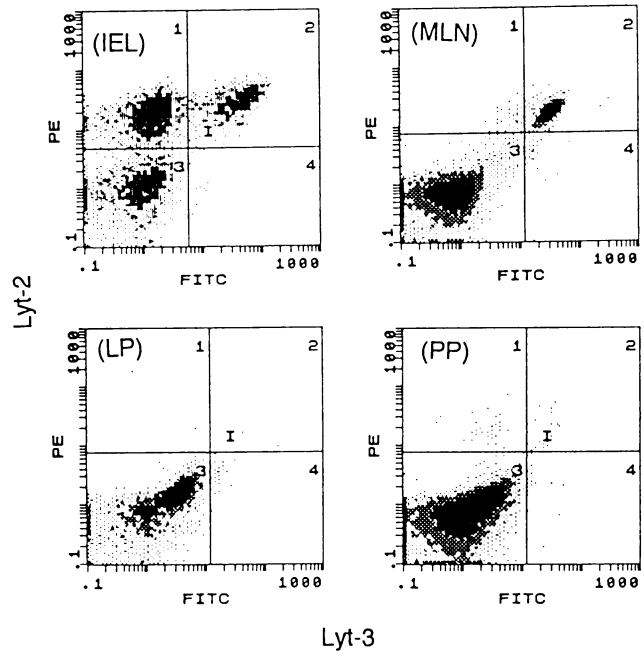

Figure 4. Flow cytometric analysis of mononuclear cells in IEL, MLN, LP and PP of mice 14 days after conventionalization using anti-Lyt-2 and anti-Lyt-3 mAb.

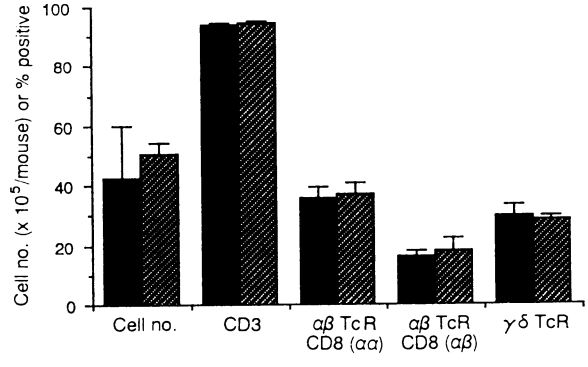

Figure 5. Effect of thymectomy on each phenotype of IEL. GF mice (12 weeks old) were thymectomized under sterile conditions immediately after removal from the vinyl isolator. Approximately 2–3 hr after the operation, the mice were conventionalized as described in Fig. 1. One month after conventionalization, each phenotype was analysed by flow cytometry. As a control, a sham operation was performed at the same time as thymectomy. Each bar represents the mean value + SD ($n = 5$). Thymectomized (■) and sham-operated (▨) mice.

the composition of each phenotype of IEL were examined. There was no difference in the per cent and number of $\alpha\beta$ and $\gamma\delta$ IEL between thymectomized and sham-operated conventionalized ex-GF mice (Fig. 5). There was also no difference in each population of IEL between these operated mice and age-matched non-operated 28 day conventionalized mice (see Fig. 1).

DISCUSSION

There was a great difference in the number of $\alpha\beta$ IEL but no difference in that of $\gamma\delta$ IEL between GF and CV mice, as described elsewhere.[8] We have clarified here that $\alpha\beta$ IEL began to increase 4 days after microbial colonization in GF adult mice, and the number of $\alpha\beta$ IEL reached the same level in CV mice approximately 1 month after conventionalization. Although the major fraction of IEL that increased after microbial association was $\alpha\beta$ IEL with a CD8 α homodimer, $\alpha\beta$ IEL with a CD8 $\alpha\beta$ heterodimer also responded to this stimulus. The kinetics of the response of $\alpha\beta$ IEL after microbial colonization in GF mice were different from those of $\alpha\beta$ T cells in GALT such as PP, MLN and LP lymphocytes. The increase in $\alpha\beta$ IEL was very slow compared to those in GALT. In particular, $\alpha\beta$ IEL with the CD8 α homodimer is a unique population, being absent in GALT. It is, therefore, assumed that there are great differences in the origin and the mechanism underlying the response to microbial association between IEL and T cells in GALT.

The increase in $\alpha\beta$ IEL after microbial association in thymectomized mice as described here, and the absence of $\alpha\beta$ IEL in athymic nude mice[2,3] suggest two possibilities. One is that the thymus-derived progenitors of $\alpha\beta$ IEL differentiate outside the thymus then repopulate the intraepithelial space in the intestine. The other is that such progenitors are present in the intraepithelial space in the intestine and proliferate there in

response to the stimuli produced by microbial association. At present, we cannot state which possibility is plausible. Guy-Grand *et al.*[2] suggested the thymus-independent differentiation of αβ IEL, because the negative selection of Vβ3 and Vβ11, and Vβ6 did not occur in BALB/c mice (Mls-1 $^{b}2^{a}$, I-E^{d}) and AKR/J mice (Mls-1 $^{a}2^{b}$), respectively. Their results and our results support that both selection involving the TcR and expansion would occur outside the thymus. However, the problem remains of where the αβ TcR IEL differentiate and expand outside the thymus. The expansion of unusual CD4/CD8 double negative αβ TcR T cells was found in the liver on intraperitoneal injection of bacteria.[12] Therefore, we cannot exclude the possibility that double negative αβ T cells respond in the liver or an organ other than the thymus then migrate into the intraepithelial space in the intestine to express CD8 molecules. Guy-Grand *et al.* have demonstrated the presence of RAG-1 mRNA, which is required for TcR rearrangement in CD3 negative IEL.[13] We have obtained evidence that both αβ and γδ IEL could be reconstituted by their transfer into scid mice (Y. Umesaki, H. Setoyama, S. Matsumoto and Y. Okada, unpublished data). These data suggest that αβ IEL expand from their progenitors in the intraepithelial space in the intestine. As to the CD8 molecules of αβ IEL, we could not find a clear difference in the response to microbial colonization in GF mice between the α homodimer and the αβ heterodimer. Both types of CD8-bearing IEL responded to the microbial association and were not affected by thymus deprivation. The results of thymectomy suggest that the thymus has no effect on the process of the expansion of αβ IEL. It was reported that αβ IEL with the CD8 αβ heterodimer, but not those with the CD8 α homodimer, were found to undergo negative selection on Vβ chain repertoire analysis.[6] Accordingly, αβ IEL with the CD8 αβ heterodimer are expected to expand outside the thymus after negative selection in the thymus.

It is also interesting to discover what evokes the IEL response in the microbial association process. Antigens of associated bacteria are candidates for the stimulus. We observed the translocation of Enterobacteriaceae and lactobacilli into MLN within 7 days of microbial association. However, since the expansion of αβ T cells with a different phenotype from that of IEL occurs on intraperitoneal injection of such bacteria, it is unlikely that IEL would expand outside the thymus in response to the translocated bacteria then migrate to the intestine. Another possibility is that the intestinal epithelial cells altered by microbial association induce the IEL response in the intraepithelial space. We have already observed the fucosylation of cell-surface glycolipids[14,15] and major histocompatibility complex (MHC) class II molecules[10] from 1 to 14 days and from 7 to 14 days, respectively, after microbial association with GF mice. Lefrancois and Goodman reported MHC I-E molecule-dependent selection of γδ TcR-bearing IEL.[16] Processed bacterial antigen in epithelial cells, if present, may be involved in this phenomenon. From the viewpoint of the function of IEL, it is assumed that γδ IEL, a major population in GF mice, play an important role in the structural change of the intestinal mucosa, such as the expression of MHC molecules in the intestinal epithelial cells and αβ IEL expansion on interferon-γ (IFN-γ) production,[11] in the early phase of microbial association, and that expanded αβ IEL play a role in the mucosal defence mechanism, for example, by destroying the injured epithelial cells in the context of MHC molecules expressed on them.[17]

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